Toxicity of Glucosinolate Degradation Products from *Brassica napus* Seed Meal Toward *Aphanomyces euteiches* f. sp. *pisi*

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ABSTRACT

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Brassica tissues are potentially useful in the control of *Aphanomyces* root rot of peas (*Pisum sativum*), but identity of the responsible compounds and specific impacts of those compounds on the pathogen's infection potential remain uncertain. *Brassica napus* seed meals and water extracts from these meals were used to determine the effect of glucosinolate hydrolysis products on *Aphanomyces euteiches* f. sp. *pisi*. *B. napus* meal ('Dwarf Essex') containing glucosinolates and intact myrosinase, the enzyme responsible for glucosinolate hydrolysis, completely inhibited infection by *A. euteiches* f. sp. *pisi* oospores. Water extracts from this meal, likewise, severely inhibited infection by oospores, as well as mycelial growth. Extracts from autoclaved 'Dwarf Essex' meal, in which myrosinase was denatured, and a low glucosinolate *B. napus* variety

('Stonewall') produced little disease reduction and had less impact on mycelial growth. Gas chromatographic analysis of *Brassica* tissues and water extracts confirmed that glucosinolates remained in autoclaved 'Dwarf Essex' meal and that 'Stonewall' meal contained low glucosinolate concentrations. 5-Vinyloxazolidine-2-thione was identified by mass spectrometry as a dominant glucosinolate hydrolysis product in aqueous extracts of the inhibitory meal. Bioassays conducted with aqueous solutions of this compound reduced mycelial growth, but not to the extent of those from intact 'Dwarf Essex' meal. Water-soluble compounds produced from the hydrolysis of glucosinolates in *B. napus* tissues reduced *A. euteiches* oospore infection and inhibited mycelial growth, thus, demonstrating potential utility of *Brassica* species in the control of *A. euteiches*.

Additional keywords: allelochemicals, rapeseed meal.

Glucosinolates, organic anions containing β -D-thioglucose and sulfonated oxime moieties, are present in Brassicaceae (Cruciferae) tissues. Hydrolysis by the enzyme myrosinase (thioglucoside glucosylhydrolase, EC 3.2.3.1) releases numerous compounds including isothiocyanates, thiocyanate ion, nitriles, and epithionitriles (5,19,34,35). The types of compounds produced are specific to the respective glucosinolates present in the tissue and conditions under which hydrolysis occurs (12,21).

It has been demonstrated that Brassicaceae residues are fungicidal (1,4,11,36) and that soil amendment with cruciferous plants reduces the incidence of *Aphanomyces* root rot of pea (4,26). The responsible mechanism for this toxicity is unclear, but the detrimental effect of cruciferous tissues on other microorganisms has been attributed mainly to volatile degradation products of glucosinolates released from these plants (7,9,10,19,20). For example, volatile compounds from macerated *Brassica* root tissue inhibited the fungal pathogen of wheat, *Gaeumannomyces graminis* (1). The glucosinolate product allyl isothiocyanate released from macerated cabbage tissues proved to be toxic toward *Peronospora parasitica* (13), and isothiocyanates and aldehydes in solarized soil amended with cabbage residues correlated with the reduction of *Pythium ultimum* and *Sclerotium rolfsii* propagules (11).

Less is known about the role of low volatility, water-soluble glucosinolate degradation products in disease suppression, but these and other water-soluble secondary compounds have been implicated in allelopathic interactions (3,22,23,31). Aqueous extracts of cabbage (*Brassica oleraceae*) did not suppress *Aphanomyces* root rot of peas (28), nor did aqueous extracts inhibit

growth of *Propionibacterium* (32). However, fungal inhibition by aqueous extracts from *Brassica* plants has been observed (27,36). Both water-soluble and volatile glucosinolate degradation products may, therefore, be involved in disease suppression and may be useful in the biological control of plant pathogens. Effects will depend on the target organism and the particular glucosinolate profile in the plant tissue.

Our objectives were to compare antifungal activities of rapeseed meal with that of aqueous rapeseed meal extracts toward *Aphanomyces euteiches* Drechs. f. sp. *pisi* W.F. Pfender & D.J. Hagedorn, and to test the hypothesis that glucosinolate degradation products, particularly those that are water-soluble, were responsible for fungicidal activity.

MATERIALS AND METHODS

Organisms and plant tissues. *A. euteiches* f. sp. *pisi* obtained from W. J. Kaiser (USDA-ARS, Pullman, WA) was maintained on Difco potato dextrose agar (PDA) (Difco Laboratories, Detroit) at 4°C. The pea (*Pisum sativum* L.) cultivar Columbia was used in all bioassay experiments, and defatted rapeseed meal from two cultivars of *Brassica napus* L. served as the source of glucosinolates. Defatted seed meal consists of high protein seed tissue remaining after oil is expressed (30). Defatted seed meal from the Dwarf Essex cultivar contains high glucosinolate concentrations, whereas meal from the Stonewall cultivar contains substantially lower glucosinolate concentrations (Table 1). 'Dwarf Essex' meal was also treated using two methods to reduce concentrations of glucosinolate hydrolysis products. The first method was to autoclave the meal at 121°C for 20 min to denature myrosinase and prevent catalysis of the glucosinolate hydrolysis reaction. An alternative treatment was to incubate the meal for 48 h in water (3:1, wt/wt) prior to use in bioassays to promote glucosinolate

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hydrolysis. Volatile and unstable glucosinolate hydrolysis products were allowed to dissipate during the incubation period, and water-soluble glucosinolate products were removed at the end of incubation by washing the meal under vacuum with deionized water. Thus, four treatments were created: intact 'Dwarf Essex' meal containing its full complement of glucosinolates and myrosinase, and three control tissues for comparison consisting of low glucosinolate 'Stonewall'; autoclaved 'Dwarf Essex' with glucosinolates, but denatured myrosinase; and 'Dwarf Essex' meal in which glucosinolates had been hydrolyzed and the volatile products removed (Table 1).

Analysis of glucosinolates and glucosinolate hydrolysis products. Glucosinolate measurement was performed using a gas chromatographic (GC) method similar to that recommended by the Canadian Grain Commission (3,6). Glucosinolates were extracted with methanol from 0.2 g of plant tissue. The extract was placed on columns of DEAE Sephadex A-25 (Sigma Chemical Co., St. Louis) that were first treated with 6 M imidazole in 5 M formic acid and rinsed with water. Other compounds were separated from the sample in rinse steps using 67% methanol and water. Glucosinolates were desulfated overnight, removed from the columns using 60% methanol, and silylated in acetone. Silylated derivatives were separated and identified using a Hewlett-Packard 5890 series II GC equipped with a 5972 quadrupole mass selective detector (MSD) (Hewlett-Packard Co., Palo Alto, CA). GC-MSD operating conditions have been previously described (3) and included the following: injector 260°C; interface 320°C; initial oven temperature 130°C for 1 min, ramped 15°/min to 320°C and held for 10 min; purge (splitless injection) 0.5 min; He flow of 1.14 ml/min; emission at 50 µA; repeller at 30 V; and scan range of *m/z* 25-470. The column coating was 5% phenyl-substituted methylpolysiloxane (HP-5MS; 30 m, 0.25 mm inside diameter, and 0.250-µm film).

Meal samples were extracted with dichloromethane (CH₂Cl₂) 20 min after wetting to determine an overall profile of glucosinolate degradation products. For most glucosinolate degradation products, analysis was done according to procedures already described (2) with a slight modification of temperature programming for the GC as follows: injector 220°C, flame ionization detector (FID) 280°C, initial oven temperature 36°C for 3 min, first ramp 20°/min to 96°C, second ramp 8°/min to 216°C, and third ramp 16°/min to 280°C and held for 7 min. In a few cases in which peak identification on FID chromatograms was difficult, relative product quantities were determined by direct comparison with phenyl isothiocyanate (internal standard) using GC-MSD chromatograms. To determine water-soluble products (except SCN⁻) in aqueous extracts, 1 ml of 0.4 M CaCl₂ was added to a 4-ml aliquot of the 1,000 ml of extract (described below). Eight milliliters of

TABLE 1. Glucosinolate concentrations (µmol/g of meal) in different types of *Brassica napus* meal

Glucosinolate	Intact 'Dwarf Essex' ^w	Autoclaved 'Dwarf Essex' ^x	Hydrolyzed 'Dwarf Essex' ^y	Intact 'Stonewall' ^z
Allyl	0.3	0.2	0.0	<0.1
3-Butenyl	37.9	36.4	0.1	1.3
4-Pentenyl	12.8	12.5	0.0	0.8
2-OH-3-butenyl	83.2	80.4	0.0	2.6
2-OH-4-pentenyl	6.7	6.5	0.0	0.2
4-Methylthiobutyl	0.9	1.0	0.0	<0.1
Phenylethyl	2.5	1.9	0.0	<0.1
5-Methylthiopentyl	0.7	0.9	0.0	<0.1
3-Indolylmethyl	0.2	0.0	0.0	0.0
4-OH-3-indolylmethyl	5.1	4.0	0.0	2.5

^w Meal contained glucosinolates and functional myrosinase enzyme responsible for glucosinolate hydrolysis.

^x Myrosinase was denatured by autoclaving.

^y Glucosinolates hydrolyzed prior to analytical measurement.

^z Low glucosinolate containing variety.

CH₂Cl₂ were then added, samples were placed on a shaker for 15 min, and analysis conducted in the same manner as for other extracts. All values presented for chemical analyses are averages of triplicate replications.

Identification of compounds was verified using GC-MSD. Operating conditions were as previously described (3) and included the following: injector 225°C, interface 280°C, initial oven temperature 36°C for 2 min, first ramp 15°/min to 96°C, second ramp 8°/min to 216°C, third ramp 18°/min to 270°C and held for 4 min, purge 0.5 min, He flow of 1.14 ml/min, emission at 50 µA, repeller at 30 V, and scan range of *m/z* 30-280. The column was an HP-5MS (30 m, 0.25 mm inside diameter, 0.250-µm film).

Oospore infection in the presence of *B. napus* meal. Bioassays were conducted in a growth chamber at 20°C using sand amended with 1% rapeseed meal (wt/wt), except in the case of the control, which contained only sand. The amended or unamended sand (33 g) was poured into 45-ml plastic tubes and wetted with 10 ml of water. Oospores were produced on a V8 juice medium (8), added as a suspension in an amount corresponding to 100 oospores/g of sand, and covered with an additional 2 to 3 mm of sand. The following day, pea seeds were placed into the tubes and lightly covered with additional sand. The tubes were top-watered for 28 days, at which time the plants were removed and the roots washed and rated on a 0 to 4 scale for *Aphanomyces* root rot (29). Each treatment was performed with 20 replicate tubes, and the entire experiment was repeated twice. Wet weights of whole plants and their respective root systems were obtained for each plant.

Oospore infection in the presence of aqueous extracts from *B. napus* meal. Extracts of water-soluble glucosinolate products were obtained at 25 ± 3°C by adding 1,000 ml of water to 30 g of

TABLE 2. Glucosinolate degradation products (µmol/g of meal) in *Brassica napus* meal

Glucosinolate degradation products ^u	Intact 'Dwarf Essex' ^v	Autoclaved 'Dwarf Essex' ^w	Hydrolyzed 'Dwarf Essex' ^x
3-Butenyl CN	0.17	0.10	0.00
1-Methylethyl ITC	Trace ^y	0.00	0.00
4-Pentenyl CN	0.04	Trace	0.00
Allyl ITC	0.02	Trace	Trace
1-Methylpropyl ITC	0.01	0.00	0.00
2-Hydroxy-3-butenyl CN	0.57	0.24	0.25
3-Butenyl ITC	4.16	Trace	0.25
Butyl ITC	0.00	Trace	Trace
2-Methylbutyl ITC	0.00	0.01	Trace
4-Pentenyl ITC	1.49	0.00	0.01
3-Butenylepithio CN	1.01	0.00	0.10
4-Methylpentyl ITC	0.02	0.00	0.00
5-Hexenyl ITC	Trace	0.00	0.00
2-Hydroxy-3-butenylepi CN1	0.79	0.00	0.07
Phenylethyl CN	0.13	0.01	0.04
2-Hydroxy-3-butenylepi CN2	0.84	0.00	0.07
4-Pentenylepithio CN	0.04	0.00	0.04
5-Methylhexyl ITC	Trace	0.00	0.00
5-Methylthiopentyl CN	0.01	0.00	0.00
4-Methylthiobutyl ITC	0.09	0.00	0.00
Phenylethyl ITC	0.65	Trace	0.07
5-Vinyl OZT	3.94	0.59	2.83
5-Methylthiopentyl ITC	0.05	0.00	0.00
5-Allyl OZT	0.67	0.00	0.36
Dimethylsulfone ^z	Trace	Trace	0.00

^u CN = nitrile, ITC = isothiocyanate, and OZT = oxazolidine-2-thione.

^v Meal contained glucosinolates and functional myrosinase enzyme responsible for glucosinolate hydrolysis.

^w Myrosinase was denatured by autoclaving.

^x Glucosinolates hydrolyzed and meal washed prior to product extraction.

^y Trace <0.01 µmol/g meal and/or detected with gas chromatographic-mass selective detector.

^z Tentative identification and, in this case, not necessarily a glucosinolate degradation product.

B. napus meal and mixing every 1 to 2 h. After 24 h, a clear aqueous extract was obtained by centrifuging the mixture. Bioassays to determine infection of *P. sativum* by *A. euteiches* oospores were conducted in a growth chamber at 20°C using sand in the same manner as those involving meal amendment. Sand (33 g) was poured into 45-ml plastic tubes and wetted with 10 ml of the meal extract. Oospores were added as a suspension in an amount corresponding to 100 oospores/g of sand and covered with an additional 2 to 3 mm of sand. The following day, pea seeds were placed into the tubes and lightly covered with additional sand. The tubes were top-watered for 18 days, at which time plants were removed and the roots washed and rated on a 0 to 4 scale for *Aphanomyces* root rot (29). Each treatment consisted of 20 replications, and the entire experiment was repeated. Wet weights of whole plants and their respective root systems were obtained for each plant.

Mycelial growth in the presence of water extracts from *B. napus* meal. Aqueous extracts of *B. napus* meal were obtained as previously described. This extract and a 50% dilution of the extract were used in place of water for the preparation of a 50% corn meal agar medium (CMA Y2). Since the agar medium was to be autoclaved, water extracts obtained from 'Dwarf Essex' meal were autoclaved to check for compound stability. After autoclaving the media, discs of the fungus from 5- to 7-day-old colonies were placed in the center of petri plates. Radial growth was measured daily on plates incubated for 5 days at 25°C. Each treatment consisted of three or four replications, and the entire experiment was performed twice.

Mycelial growth in the presence of 5-vinylloxazolidine-2-thione. Glucosinolate analyses showed 2-hydroxy-3-butenyl to be the predominant glucosinolate in *B. napus* meal (Table 1). The hydrolytic product of this glucosinolate is an isothiocyanate that spontaneously cyclizes to the water-soluble compound 5-vinylloxazolidine-2-thione. This compound was predicted as the major glucosinolate product in meal extracts and, therefore, used in bioassays to determine its biological activity. 5-Vinylloxazolidine-2-thione (Lancaster Synthesis Ltd., Windham, NH) was dissolved in distilled water, passed through a 0.2-µm membrane filter (Nalge Co., Rochester, NY), and added to melted CMA Y2 agar to achieve a final concentration of 1.48 µmol/cm³. This concentration of 5-vinylloxazolidine-2-thione was identical to that present in the CMA Y2 agar prepared with aqueous extracts from *B. napus* meal. A 5-mm disc of *A. euteiches* was placed in the center of the

petri plate containing CMA Y2 prepared with 5-vinylloxazolidine-2-thione, and the plates were incubated under lighted conditions at 25°C. The diameter of the colonies was measured daily and compared with control plates containing CMA Y2 without 5-vinylloxazolidine-2-thione. Each treatment was replicated four times, and the experiment was repeated.

RESULTS

Analysis of glucosinolates and glucosinolate hydrolysis products. Intact *B. napus* meal contained functional myrosinase and four principal glucosinolates including 2-hydroxy-3-butenyl, 3-butenyl, and 4-pentenyl (Table 1). Both autoclaved meal, in which myrosinase was denatured, and intact meal contained all glucosinolates at similar concentrations. Prior addition of water to the intact meal effectively eliminated glucosinolates in the meal designated as hydrolyzed. Glucosinolate analysis of the 'Stonewall' meal confirmed the presence of low glucosinolate concentrations (Table 1).

Glucosinolate hydrolysis products in CH₂Cl₂ extracts of wetted meal reflect substrate and enzyme differences among the tissues (Table 2). Intact 'Dwarf Essex' meal produced a number of glucosinolate hydrolysis products in excess of 1 µmol/g of meal including 3-butenyl isothiocyanate, 5-vinylloxazolidine-2-thione, 4-pentenyl isothiocyanate, and 3-butenyl epithionitrile. In contrast, the lack of functional myrosinase resulted in very little glucosinolate hydrolysis in autoclaved meal, despite the presence of substantial glucosinolate concentrations. The only glucosinolate hydrolysis product extracted in excess of 1 µmol/g of meal from previously hydrolyzed meal was the relatively nonvolatile 5-vinylloxazolidine-2-thione.

In contrast to meal, aqueous extracts of intact 'Dwarf Essex' were dominated by 5-vinylloxazolidine-2-thione (49.5 µmol/g of meal) (Table 3). Other less water-soluble products such as isothiocyanates and nitriles were present at less than 1.5 µmol/g of meal. Extracts from autoclaved 'Dwarf Essex' and intact 'Stonewall' meals also contained 5-vinylloxazolidine-2-thione as the major

TABLE 3. Concentrations (µmol/g of meal) of glucosinolate degradation products in aqueous extracts of *Brassica napus* meal

Glucosinolate degradation products ^u	Extract from intact	Extract from autoclaved	Extract from	Autoclaved extract from intact
	'Dwarf Essex' ^v	'Dwarf Essex' ^w	'Stone wall' ^x	'Dwarf Essex' ^y
3-Butenyl CN	0.10	0.06	0.00	0.04
2-Hydroxy-3-butenyl CN	0.56	0.30	0.01	0.53
3-Butenyl ITC	0.17	0.00	0.02	Trace ^z
3-Butenylepithio CN	1.29	0.00	0.06	0.98
2-Hydroxy-3-butenylepi CN1	0.71	0.00	0.01	0.51
2-Hydroxy-3-butenylepi CN2	1.02	0.00	0.00	0.93
5-Vinyl OZT	49.48	1.06	1.80	46.35
5-Allyl OZT	4.90	0.00	0.00	4.46
4-Methylsulfinylbutyl ITC	~0.42	0.00	0.00	~0.07
5-Methylsulfinylpentyl ITC	~0.39	0.00	0.00	~0.09

^u CN = nitrile, ITC = isothiocyanates, and OZT = oxazolidine-2-thione.

^v Meal from which extract was obtained contained glucosinolates and functional myrosinase enzyme responsible for glucosinolate hydrolysis.

^w Myrosinase in meal was denatured by autoclaving prior to product extraction and analytical measurement.

^x Glucosinolates hydrolyzed and meal washed prior to product extraction.

^y Aqueous extract obtained from meal containing glucosinolates and functional myrosinase was autoclaved.

^z Trace <0.05 µmol/g of meal.

TABLE 4. *Aphanomyces euteiches* f. sp. *pisi* oospore infection in the presence of 1% amendments of *Brassica napus* meal to sand as determined 28 days after inoculation

Treatment ^t	Aphanomyces rating (0-4) ^u	Total plant weight ^v (g)	Root weight (g)
Without pathogen			
No meal	0.0	1.53 a	0.40 a
'Dwarf Essex' ^w	0.0	1.44 ab	0.30 b
Autoclaved 'Dwarf Essex' ^x	0.0	1.34 b	0.31 b
Hydrolyzed 'Dwarf Essex' ^y	0.0	1.43 ab	0.27 b
'Stonewall' ^z	0.0	1.39 ab	0.33 b
With pathogen			
No meal	2.4 a	1.41 a	0.32 b
'Dwarf Essex'	0.0 c	1.41 a	0.30 ab
Autoclaved 'Dwarf Essex'	1.1 b	1.20 b	0.24 bc
Hydrolyzed 'Dwarf Essex'	0.6 bc	1.36 ab	0.25 bc
'Stonewall'	2.2 a	1.24 ab	0.23 c

^t Each treatment was performed by adding 100 oospores/g of sand followed in 24 h by pea seed addition.

^u Overall means between inoculated and uninoculated treatments were different ($P < 0.05$). Means within either an inoculated or uninoculated treatment and in the same column followed by the same letter are not statistically different ($P > 0.05$) according to the least significant difference test. Data represent the mean of 16 to 20 replicates per treatment. The experimental data represent the results of a single experiment. The experiment was repeated and similar results were obtained.

^v All reported plant and root weights were fresh weight measurements.

^w Meal contained glucosinolates and functional myrosinase enzyme responsible for glucosinolate hydrolysis.

^x Myrosinase was denatured by autoclaving prior to meal amendment.

^y Glucosinolates hydrolyzed and products removed prior to meal amendment.

^z Low glucosinolate containing variety.

product, but at less than 2.0 $\mu\text{mol/g}$ of meal. Autoclaving water extracts obtained from 'Dwarf Essex' meal did not substantially alter concentrations of most observed glucosinolate hydrolysis products (Table 3). Therefore, autoclaved agar containing these extracts was, likewise, expected to retain the respective glucosinolate products at bioassay initiation.

Oospore infection in the presence of *B. napus* meal. Intact 'Dwarf Essex' meal with its high concentration of glucosinolates and intact myrosinase completely suppressed oospore infection (Table 4). Autoclaved and hydrolyzed 'Dwarf Essex' meal with reduced glucosinolate concentrations also decreased *Aphanomyces* root rot compared with the infested control ($P < 0.05$), but not to the extent of the intact meal. 'Stonewall' meal with its low concentration of glucosinolates did not reduce oospore infection as compared with the infested control (Table 4).

The impact of *Aphanomyces* infestation on pea plant weights was also determined in the presence and absence of various meal amendments. Uninfested meal-treated soils produced total plant weights similar to the uninfested control, except in one treatment (Table 4). Root weights were reduced below that of the respective unamended controls by meal amendment to uninfested treatments and most of the infested treatments. Total plant weights were reduced below that of the respective uninfested and infested controls only with the addition of autoclaved 'Dwarf Essex' meal.

Oospore infection in the presence of aqueous extracts from *B. napus* meal. Water extracts from intact 'Dwarf Essex' meal almost completely inhibited development of *Aphanomyces* root rot disease in bioassay experiments (Table 5). Extracts from autoclaved 'Dwarf Essex' meal and extracts from 'Stonewall' meal were ineffective in reducing the disease. Although these two extracts differed from each other statistically, they did not differ ($P > 0.05$) from the infested control. In contrast to experiments with meal amendments, extracts from autoclaved 'Dwarf Essex' meal showed no toxicity toward oospores.

Root weights were not altered in the presence of water extracts from intact 'Dwarf Essex' meal, but were lowered in the presence of extracts from autoclaved and 'Stonewall' meals (Table 5). Total plant weights were lowered by the addition of aqueous extracts, except the infested treatment amended with an extract from intact 'Dwarf Essex' meal.

TABLE 5. Effect of aqueous extract from *Brassica napus* meal on oospore infection

Treatment ^u	<i>Aphanomyces</i> rating (0-4) ^v	Total plant weight ^w (g)	Root weight (g)
Without pathogen			
Water	0.0	1.64 a	0.42 a
'Dwarf Essex' ^x	0.0	1.48 b	0.37 ab
Autoclaved 'Dwarf Essex' ^y	0.0	1.36 bc	0.33 bc
'Stonewall' ^z	0.0	1.34 c	0.29 c
With pathogen			
Water	2.6 ab	1.40 a	0.35 a
'Dwarf Essex'	0.2 c	1.46 a	0.33 a
Autoclaved 'Dwarf Essex'	3.4 a	1.17 b	0.26 b
'Stonewall'	2.5 b	1.01 b	0.22 b

^u Each treatment was performed by adding 100 oospores/g of sand followed in 24 h by pea seed addition.

^v Overall means between inoculated and uninoculated treatments were different ($P < 0.05$). Means within either an inoculated or uninoculated treatment and in the same column followed by the same letter are not statistically different ($P > 0.05$) according to the least significant difference test. Data represent the mean of 16 to 20 replicates per treatment. The experimental data represent the results of a single experiment. The experiment was repeated and similar results were obtained.

^w All reported plant and root weights were wet weight measurements.

^x Meal contained glucosinolates and functional myrosinase enzyme responsible for glucosinolate hydrolysis.

^y Myrosinase was denatured by autoclaving prior to meal amendment.

^z Low glucosinolate containing variety.

Mycelial growth in the presence of water extracts from *B. napus* meal. Water extracts from intact 'Dwarf Essex' meal completely inhibited mycelial growth of *Aphanomyces*, even at the 50% dilution (Fig. 1). Extracts from autoclaved and 'Stonewall' meals did not reduce mycelial growth to this extent, but reduced growth significantly compared with the control ($P < 0.05$). The reduction in growth was the same for both of these extracts and was similar with both the original extracts (data not shown) and 50% dilutions of that extract (Fig. 1).

Mycelial growth in the presence of 5-vinylloxazolidine-2-thione. 5-Vinylloxazolidine-2-thione at a concentration equivalent to that measured in aqueous extracts of intact 'Dwarf Essex' meal reduced fungal growth in petri plate assays (Fig. 2). Suppression of mycelial growth was considerably less, however, than observed with aqueous extracts from intact 'Dwarf Essex' meal.

DISCUSSION

Glucosinolates (I) are hydrolyzed by myrosinase to form unstable aglucone intermediates (II) (Fig. 3). Under neutral conditions, the aglucone undergoes Lossen-type rearrangement to produce the respective isothiocyanate (III). The presence of acidic conditions, specific cofactors, or Fe^{2+} favors the production of nitriles (IV). The large number of isothiocyanates and nitriles identified in CH_2Cl_2 extracts demonstrates the prominence of these hydrolysis products in intact meal (Table 2). Hydrolysis products are absent in CH_2Cl_2 extracts of autoclaved meal because myrosinase was no longer functional, not because of reduced glucosinolate concentrations (Table 1). The elimination of glucosinolates in previously hydrolyzed meal (Table 1) explains the lack of isothiocyanates and nitriles in the respective CH_2Cl_2 extracts (Table 2). Glucosinolates were hydrolyzed and isothiocyanates and nitriles, thus, dissipated prior to extraction with CH_2Cl_2 .

As expected, isothiocyanates and nitriles were not prominent products in aqueous extracts (Table 3), because most have low solubilities. Aqueous extracts are, instead, dominated by 5-vinyl-

Fig. 1. *Aphanomyces* mycelial growth in the presence of extracts from defatted *Brassica napus* meal. Extracts of water-soluble glucosinolate products were obtained at $25 \pm 3^\circ\text{C}$ from 30 g of *B. napus* meal. A 50% dilution of the extract was used in place of water for the preparation of a 50% corn meal agar medium (CMA Y2). After autoclaving the media, discs of the fungus from 5- to 7-day-old colonies were placed in the center of petri plates and radial growth was measured daily on plates incubated for 5 days at 25°C .

oxazolidine-2-thione (VI) (Fig. 3), a compound originating from 2-hydroxy-3-butenyl glucosinolate. This glucosinolate, present at high concentrations in intact meal, produces 2-hydroxy-3-butenyl isothiocyanate (V), which spontaneously cyclizes to form the oxazolidinethione (Fig. 3). This intramolecular reaction is typical for glucosinolates possessing a β -hydroxyl group (24).

Intact 'Dwarf Essex' meal completely inhibited oospore infection in amendment experiments, most likely because of the combined impact of volatile and water-soluble allelochemicals such as isothiocyanates, nitriles, and oxazolidinethiones identified in Table 2. 'Stonewall' meal, with lower glucosinolate concentrations, did not produce these compounds and, correspondingly, did not inhibit oospore infection. The lack of antifungal activity with 'Stonewall' meal provides evidence that decreased oospore infection was not a result of organic carbon addition and a general increase in microbial activity.

The two treated forms of 'Dwarf Essex' meal, hydrolyzed and autoclaved, showed partial inhibition of oospore infection in amendment experiments, most likely because low concentrations of allelochemicals remained within the meal tissues. In hydrolyzed meal, detectable concentrations of 5-vinyloxazolidine-2-thione, 3-butenyl isothiocyanate, phenylethyl isothiocyanate, and 5-allyloxazolidine-2-thione were present (Table 2). 3-Butenyl isothiocyanate and phenylethyl isothiocyanate are among the more fungitoxic of the naturally occurring isothiocyanates (1,25).

Autoclaved tissues contained trace concentrations of isothiocyanates and nitriles and a small but measurable amount of 5-vinyloxazolidine-2-thione (Table 2), which could reduce oospore infection. Although high glucosinolate concentrations were present in these tissues, previous research indicates that it is unlikely that glucosinolates themselves altered the ability of oospores to infect the tissues. Kyung and Fleming (18) showed that antibacterial activity of cabbage (*B. oleraceae*) is destroyed by heating, and Mithen et al. (25) determined that intact glucosinolates do not display significant antifungal activity towards *Leptosphaeria*

maculans. However, fungi and bacteria produce enzymes capable of glucosinolate hydrolysis (34), and product formation could have occurred in the rhizosphere of pea plants in this experiment. Alternatively, autoclaved and hydrolyzed meal may contain toxic compounds not identified in our analyses.

The effects observed with meal amendments include the combined impact of a variety of compounds with varying degrees of volatility. Biological effects observed in research with glucosinolate-containing plant tissues are often attributed to volatile compounds (1,11,13,15,16). Our results clearly demonstrated that aqueous extracts from intact 'Dwarf Essex' meal containing less volatile compounds severely reduced oospore infection. Extracts from low glucosinolate 'Stonewall' meal and autoclaved 'Dwarf Essex' meal had no effect on infection. Mycelial growth was altered similarly, with complete inhibition in the presence of water extracts from intact 'Dwarf Essex' meal (Fig. 1). Extracts prepared from autoclaved 'Dwarf Essex' and low glucosinolate 'Stonewall' meals also decreased fungal growth compared with the control, but not to the extent of extracts from intact meal.

These results are consistent with chemical analyses in which intact meal extracts contained substantially higher allelochemical concentrations than autoclaved and 'Stonewall' extracts. The glucosinolate hydrolysis product in highest concentration in all aqueous extracts was 5-vinyloxazolidine-2-thione, with concentrations near 50 $\mu\text{mol/g}$ in extracts from intact meal, but less than 2.0 $\mu\text{mol/g}$ in autoclaved and 'Stonewall' extracts. Our results showed that 5-vinyloxazolidine-2-thione decreased mycelial growth of *A. euteiches* on petri plates, however, less effectively than aqueous meal extracts, despite the use of equivalent oxazolidinethione concentrations. Thus, toxicity of meal extracts can be only partially explained by the presence of 5-vinyloxazolidine-2-thione.

Beside the well-known ability of 5-vinyloxazolidine-2-thiones to induce goiter of the thyroid gland, general toxicity appears to be relatively low. For example, oxazolidinethiones had little effect on propionic bacteria (32) or *Leptosphaeria maculans* (25), but, similar to our case, had some effect against *Phoma lingam* (33). Concentrations of additional glucosinolate degradation products in our aqueous extracts such as 3-butenyl or methylsulfinyl isothiocyanates were relatively low, but, in contrast to studies with pure

Fig. 2. Inhibition of *Aphanomyces* mycelial growth by 5-vinyloxazolidine-2-thione. 5-Vinyloxazolidine-2-thione was added to 50% corn meal agar medium (CMA Y2) to achieve a final concentration of 1.48 $\mu\text{mol/cm}^3$. A 5-mm disc of *A. euteiches* was placed in the center of the petri plate containing CMA Y2 prepared with 5-vinyloxazolidine-2-thione, and the plates were incubated under lighted conditions at 25°C. The diameter of the colonies was measured daily and compared with control plates containing CMA Y2 without 5-vinyloxazolidine-2-thione.

Fig. 3. Glucosinolate hydrolysis products resulting from enzymatic degradation and spontaneous rearrangements. I = glucosinolate, II = aglucone, III = isothiocyanate, IV = nitrile, V = 2-hydroxy-3-butenyl isothiocyanate, and VI = 5-vinyloxazolidine-2-thione.

oxazolidinethiones, these compounds may account for the higher activity of the extracts by acting synergistically or in an additive manner with oxazolidinethione.

In addition to effects on *A. euteiches*, meal and aqueous extracts, in some cases, negatively impacted plant growth. Glucosinolate hydrolysis products are indeed phytotoxic (14). Inhibition of plant growth as reflected in lower green tissue or root weights, however, was, in many cases, independent of glucosinolate hydrolysis product concentration in the meal or meal extracts. Even 'Stonewall' meal with low glucosinolate concentrations reduced plant weights. Rapeseed meal contains a high concentrations of phenolics (17), compounds potentially detrimental to crops (23). Commonly cultivated rapeseeds including those with low levels of glucosinolates have similar phenolic concentrations (17), which may partially explain the observed phytotoxicity. Phytotoxicity is, thus, not solely a function of glucosinolate concentrations, and disease suppression with glucosinolate-containing tissues may be possible with minimal plant growth inhibition.

In summary, we demonstrated that toxicity of rapeseed meal or aqueous meal extracts toward *A. euteiches* strongly correlated with the presence of glucosinolate degradation products. Our results demonstrate that the responsible allelochemicals include not only highly volatile constituents as previously predicted, but low volatility water-soluble compounds that include, but are not limited to, 5-vinyloxazolidine-2-thione.

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